AD_____

GRANT NUMBER DAMD17-94-J-4173

TITLE: Stromal Components of Breast Cancer Progression

PRINCIPAL INVESTIGATOR: Sandra W. McLeskey, Ph.D.

CONTRACTING ORGANIZATION: Georgetown University
                          Washington, DC 20057

REPORT DATE: July 1997

TYPE OF REPORT: Annual

PREPARED FOR: Commander
               U.S. Army Medical Research and Materiel Command
               Fort Detrick, Frederick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;
                         distribution unlimited

The views, opinions and/or findings contained in this report are
those of the author(s) and should not be construed as an official
Department of the Army position, policy or decision unless so
designated by other documentation.

DTIC QUALITY INSPECTED 3

19971008 036

REPORT DOCUMENTATION PAGE		*Form Approved* *OMB No. 0704-0188*

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE July 1997	3. REPORT TYPE AND DATES COVERED Annual (15 Jun 96 - 14 Jun 97)	

4. TITLE AND SUBTITLE
Sromal Influences on Breast Cancer Progression

5. FUNDING NUMBERS
DAMD17-94-J-4173

6. AUTHOR(S)
Sandra W. McLeskey, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Georgetown University
Washington, DC 20057

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, MD 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200)*

Tumors produced in mice by native MCF-7 breast carcinoma cells or MCF-7 cells transfected with the angiogenic growth factors FGF-4, FGF-1, or VEGF were studied at early time points following tumor cell injection. Tumor cell growth or death was assessed using staining for BrdU incorporation or apoptosis under conditions of treatment with nothing, estrogen, or tamoxifen in ovariectomized mice. BrdU incorporation correlates with known hormonally-influenced growth characteristics of the tumor cells at early time points before differences in tumor size become apparent. Abundance of intratumoral blood vessels and tumor edge-associated vessels may be positively associated, and abundance of normal stromal vessels negatively associated with BrdU incorporation. Endothelial cells have been isolated from tumors produced by MCF-7 cells or the transfectants by flow cytometry utilizing monoclonal antibodies to PECAM-1. RNA produced from the isolated cells contains transcripts for PECAM-1 in greater abudance than that of EOMA cells, a murine hemangioma cell line used as a control. Transcripts for the human keratinocyte growth factor receptor expressed by the tumor cells are also present in the RNA produced from the isolated endothelial cells, indicating some degree of tumor cell contamination in the isolated cells. This RNA will be used to identify genes differentially expressed in endothelial cells participating in tumor-induced angiogenesis.

14. SUBJECT TERMS Breast Cancer, Estrogen Dependence, Stroma, Angiogenesis, Endothelial Cells, Growth Factors, FGFS			15. NUMBER OF PAGES 20
			16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

_____ Where copyrighted material is quoted, permission has been obtained to use such material.

_____ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

_____ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

SWM In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

_____ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

SWM In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

SWM In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

SWM In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Sandra W. McLesley   7/13/97
PI - Signature   Date

TABLE OF CONTENTS

INTRODUCTION

The overall purpose of this project is to study gene expression in endothelial cells participating in tumor-induced angiogenesis, with the aim of identifying characteristics of these cells which could serve as targets for cancer therapy. The model system used is an *in vivo* model of breast cancer which consists of wild type MCF-7 breast carcinoma cells or the same cells transfected with angiogenic growth factors growing as tumors in nude mice. Although the original proposal utilized MCF-7 cells transfected with fibroblast growth factor 4 (FGF-4) as the angiogenic growth factor, we have expanded the project to utilize MCF-7 cells transfected with FGF-1 (a.k.a. acidic FGF) or vascular endothelial cell growth factor (VEGF). We have expanded our study since FGF-4 has been found not to be expressed in human breast cancer, while FGF-1 and VEGF are thought to be important angiogenic factors for many types of cancer, including breast. Cells transfected with FGF-1 and VEGF are available from the laboratory of the PI's postdoctoral fellowship and utilize the same clonal line of parental cells. Therefore, we thought it important to include transfectants with these more relevant angiogenic factors in our studies.

Morphological and topographical studies have been conducted to determine the identity of blood vessels which are associated with productive tumor growth, vs blood vessels coincidentally associated with the tumor or blood vessels generated by the inflammatory response set up by tumor cell injection. RNA from endothelial cells isolated from growing tumors by fluorescence-activated cell sorting (FACS) is being purified from tumors produced by the parental, FGF, or VEGF transfected cells as well as more differentiated endothelial cells from mammary fat pads. This RNA will be utilized for RT-PCR to study gene expression of known genes important in angiogenesis, such as FGF receptors and VEGF receptors, and for differential display PCR to discover genes differentially expressed by the angiogenic endothelial cells in tumors.

BODY

Aim 1. As mentioned in last year's report, Aim 1 of this project was to validate the model of tumor-induced angiogenesis by correlating microvessel density with tumor size and/or metastasis in these experimental tumors. A positive correlation between tumor size and microvessel density was achieved in the first year using archival slides from tumors produced by FGF-4 or FGF-1 transfected cells.

Aim 2. In Aim 2, we sought to discern a difference in the temporal and spatial relationship of blood vessels present in tumors produced by FGF-4 transfected MCF-7 cells when compared with tumors produced by the parental cells. As mentioned, this aim has expanded to include FGF-1 transfected and VEGF-transfected MCF-7 cells. Over time, we have realized that the differences we are describing are really between tumors which are actively growing vs those which are static or regressing, regardless of the identity of the transfected or nontransfected cells which produced the tumors. (Tumors produced by the VEGF transfected cells have not yet been subjected to analysis and therefore, this generalization may not apply to them.)

This past year, we have been developing image analysis and morphologic techniques which will differentiate actively growing tumors from static or regressing tumor nodules at early time points in tumor development before differences in size become apparent. In work described last year, we had identified substantial inflammatory reaction which takes place immediately after tumor cell injection. Between 10 and 20 days after tumor cell injection, however, this acute inflammatory reaction disappears and tumors begin to grow actively or to regress. Therefore, we have chosen the time point of 10-20 days after tumor cell injection as our "window" for discerning differences between actively growing tumors and static or regressing ones. The techniques, described below, of measuring proliferation and apoptosis, and of describing blood vessel morphology and topography, will enable us to study gene expression in pertinent vessels in pertinent tumors and to identify genes expressed by those particular microvessels associated with productive tumor growth.

Since tumor growth is the algebraic sum of tumor cell proliferation and tumor cell death, we have evaluated both of these parameters utilizing staining for bromodeoxyuridine (BrdU) incorporation as a measure of proliferation and apoptosis (TUNEL) staining (1) as a measure of cell death. Tumor sections from tumors produced by parental or transfected cells and harvested from 10 to 20 days after tumor cell injection are stained for BrdU or TUNEL and the positively-stained nuclei are quantitated by image analysis. Areas of the section are identified which are occupied mainly by tumor and the quantitation is restricted to these areas. In addition, an area measurement is obtained. Cell density per unit area of the various tumors is determined so that a labeling fraction for each type of staining can be calculated. These studies have been done for FGF-4 and FGF-1 transfected cells as well as parental cells grown in ovariectomized mice treated with nothing, estrogen, or tamoxifen. Since the *in vivo* growth characteristics of the cells under these treatment conditions are known, the

results of these studies can be correlated with tumor growth, even though the individual tumors under study are of similar size at the time points selected. It should be pointed out that since all tumor cell injections do not result in tumors which persist after the 10-20 day time period even under the most favorable conditions, some of the individual tumors analyzed may not be behaving as predicted. However, by looking at the mean values of the labeling indices, we should be able to correlate mean values for BrdU incorporation and/or TUNEL staining with the known growth characteristics of the tumors. Thus, the purpose of these studies is to establish the validity of BrdU incorporation and/or TUNEL staining as an *in situ* predictor of tumor growth. Figure 1 shows the *in vivo* growth characteristics of 4 of the 5 cell
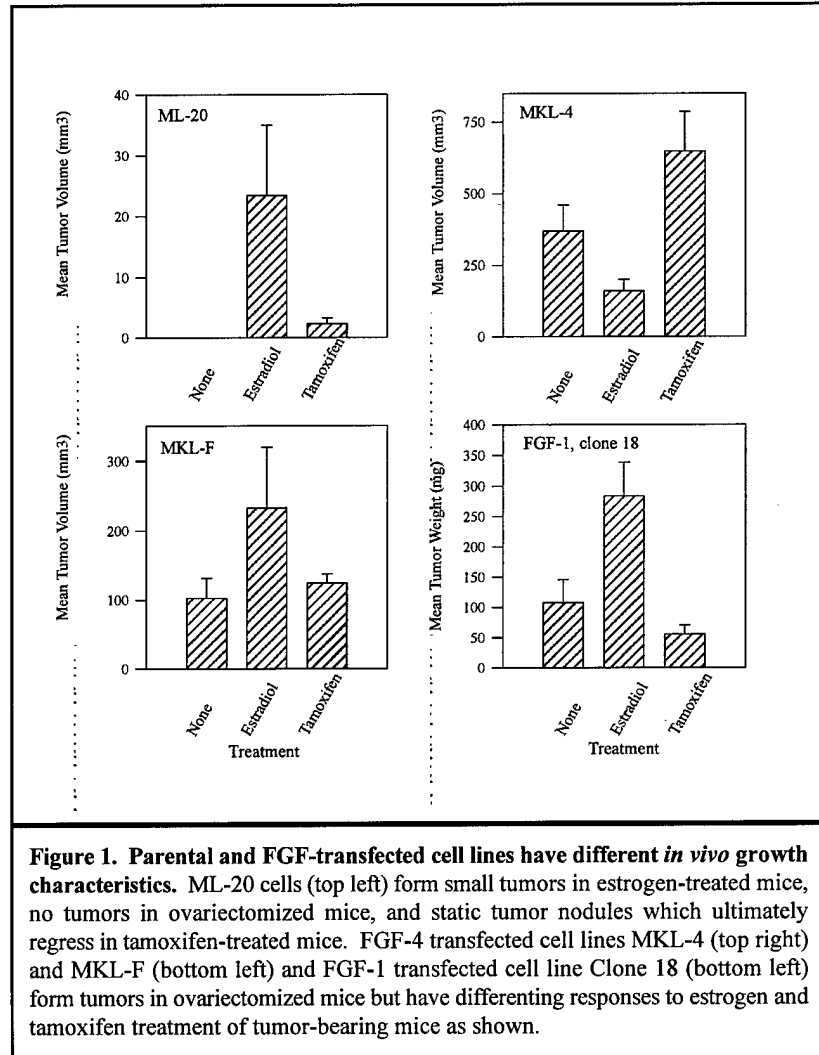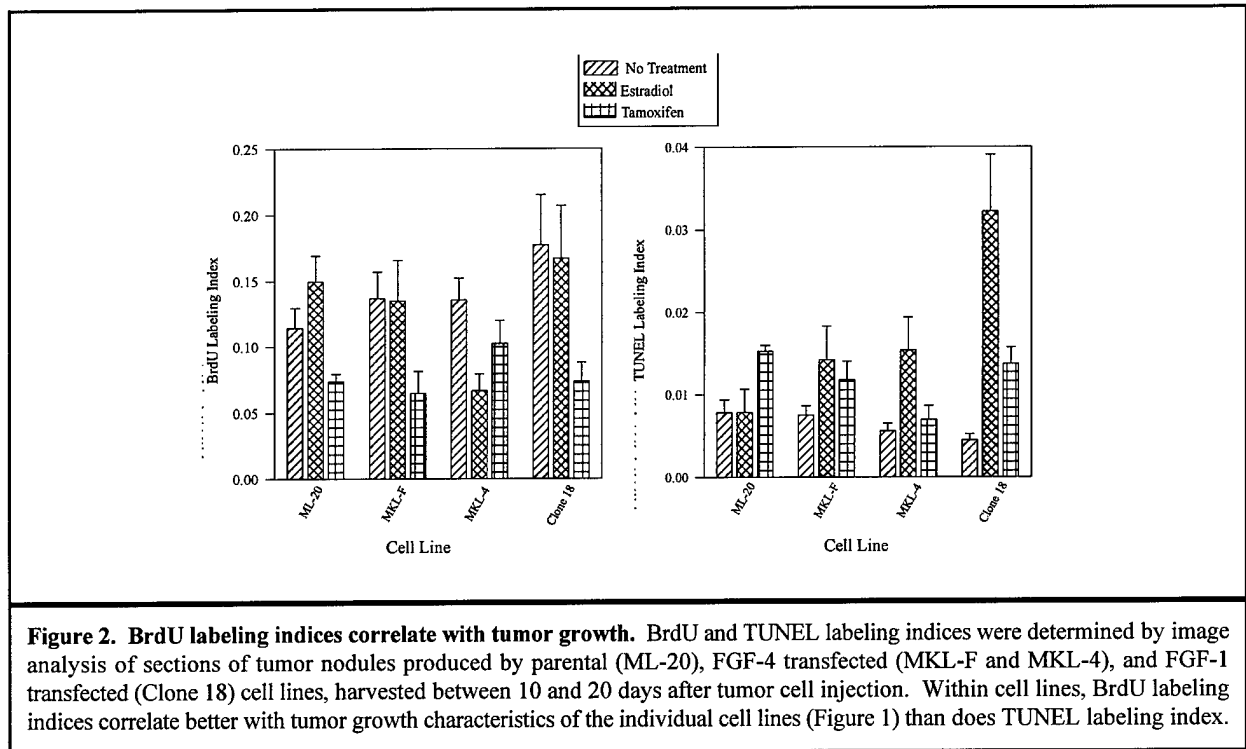

Figure 1. Parental and FGF-transfected cell lines have different *in vivo* growth characteristics. ML-20 cells (top left) form small tumors in estrogen-treated mice, no tumors in ovariectomized mice, and static tumor nodules which ultimately regress in tamoxifen-treated mice. FGF-4 transfected cell lines MKL-4 (top right) and MKL-F (bottom left) and FGF-1 transfected cell line Clone 18 (bottom left) form tumors in ovariectomized mice but have differenting responses to estrogen and tamoxifen treatment of tumor-bearing mice as shown.

lines (parental and 3 FGF transfectants) under study under conditions of the treaments with nothing, estrogen, or tamoxifen, and Figure 2 shows the mean labeling indices for TUNEL and BrdU incorporation. It would seem that within the particular cell lines, BrdU incorporation is the best *in situ* predictor of tumor growth, since it correlates best with the known *in vivo* growth characteristics of the cells. One might expect that the data from the two methods would be complementary and that either BrdU incorporation or TUNEL staining would be an accurate predictor of tumor growth. However, methodologic differences in the two methods may make one or the other method more sensitive in the particular circumstances of our study. Since BrdU incorporation is cumulative and labels all cells which have synthesized new DNA during the period of exposure to BrdU (it was injected 8 hours prior to sacrifice), there is a substantial signal present even in slowly growing or regressing tumors. TUNEL staining is much more time sensitive, in that DNA fragments are only

7

Figure 2. BrdU labeling indices correlate with tumor growth. BrdU and TUNEL labeling indices were determined by image analysis of sections of tumor nodules produced by parental (ML-20), FGF-4 transfected (MKL-F and MKL-4), and FGF-1 transfected (Clone 18) cell lines, harvested between 10 and 20 days after tumor cell injection. Within cell lines, BrdU labeling indices correlate better with tumor growth characteristics of the individual cell lines (Figure 1) than does TUNEL labeling index.

labeled by the terminal transferase enzyme for a short period during the cell's progression through the apoptotic process (1). The number of cells labeled by TUNEL is very small (1-3%) in our sections, making differences between sections difficult to discern. At any rate, since BrdU incorporation seems to be the best *in situ* predictor of tumor growth in our model, we have established mean values for BrdU incorporation tumors produced by the parental and FGF transfected cell lines for actively growing vs static or regressing tumors which can be used to evaluate the growth status of such tumors in the future.

The VEGF transfected cells produce tumors in estrogen-treated animals which are somewhat larger than those produced by the parental cells. When injected with the matrix material, Matrigel, these transfectants also produce tumors in tamoxifen-treated animals, while the parental cells do not (unpublished). Thus, the growth differences between these transfectants and the parental cells are more subtle those those produced by FGF transfection. This may make correlation between BrdU or TUNEL staining more difficult to establish in these transfectants. This effort is currently underway.

Because of initial studies in which we noticed differences in morphology and topography of tumor-associated blood vessels in growing tumors vs static or regressing ones in tumors produced

by the parental or FGF transfected cells, described last year, we have extended our study to semiquantitatively describe the morphologic and topographical characteristics of tumor-associated blood vessels in sections from tumors produced by parental or transfected cells under all conditions of treatment. We have evaluated the abundance of four types of blood vessels: 1. Intratumoral blood vessels, 2. Edge-associated blood vessels, 3. Normal stromal blood vessels, and 4. Ectatic stromal blood
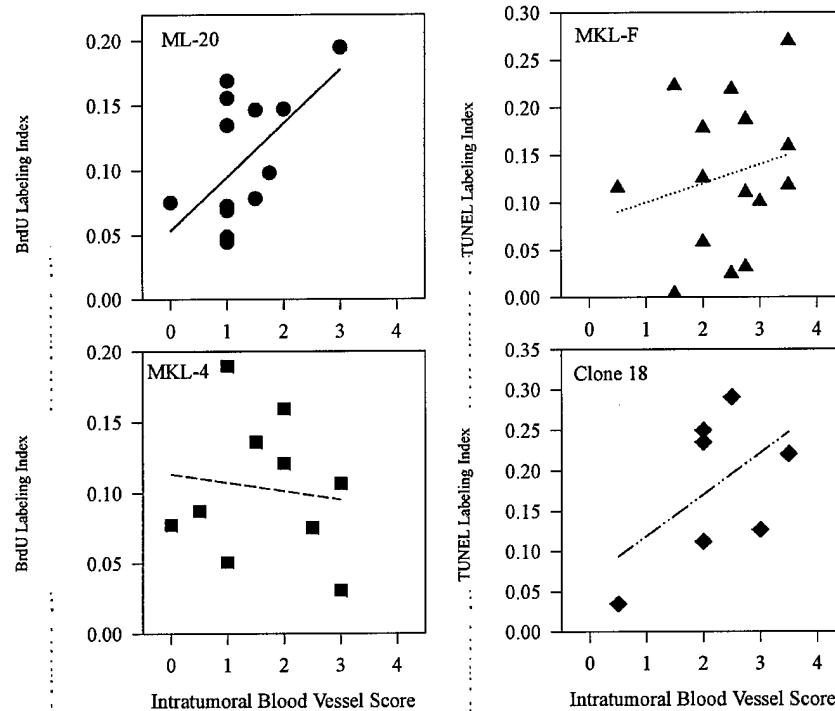

Figure 3. BrdU incorporation and intratumoral blood vessel abundance scores are positively correlated for 3 of the 4 cell lines tested. Intratumoral blood vessels stained for PECAM were rated in sections which were adjacent to those used for BrdU incorporation and TUNEL staining (Figure 2). The rating system was on a scale from 0-4, with 4 being most abundant. The rater was blinded as to the identity of the tumor and instructed not to take tumor size into consideration during the rating.

vessels. Each blood vessel type was evaluated for abundance on a scale of 0-4, with 4 being very abundant. The initial rater was a pathology resident who was blinded as to the identity of the tumor and was instructed not to take tumor size into consideration during the rating process. Ratings for the different types of vessels were then correlated with BrdU incorporation or TUNEL labeling indices for the same tumors. The results of the correlation for intratumoral blood vessels are shown in Figure 3. This figure shows that intratumoral blood vessel abundance is positively correlated with BrdU labeling index for all cell lines analyzed except MKL-4. We also find edge-associated blood vessel abundance to be positively correlated with BrdU labeling index for all cell lines except MKL-4, normal stromal vessel abundance to be negatively correlated with BrdU labeling index for all cell lines except ML-20, and stromally located, ectatic vessel abundance to be of uncertain significance. We are in the process of adding additional tumor samples to this analysis, which we hope will enable

9

us to show uniform correlations across all cell lines. In addition, we are adding the VEGF transfected cells, which we hope will also show the same correlations. In addition, we will utilize a second rater for all the sections, which will hopefully validate the first rater's findings and add statistical power to our analysis. These strategies should improve the chances that the correlations will be uniform and achieve significance. When the analysis is complete, John Hanfelt, PhD, a statistician with the Lombardi Center Statistics Core facility will provide statistical analysis of the various correlations. We envision submitting a manuscript containing this data in early fall of this year.

Aim 3. As mentioned in last year's report, a major aim of the project has been isolation of tumor-derived endothelial cells by FACS. Last year, we reported that we had isolated 100,000 cells from a tumor using two different FITC-coupled rat monoclonal antibodies to the murine endothelial cell adhesion molecule PECAM-1. The isolated cells were capable of uptake of fluorescently-tagged LDL, a property of endothelial cells. During this past year, we have been engaged in further development of semiquantitative RT-PCR assays to demonstrate the endothelial origin of RNA isolated from these cells as well as the presence of contaminating tumor or stromal cells. These assays utilize ^{35}S-labeled nucleotides in the PCR reactions and produce radiolabeled bands when the products of the reaction are run on a polyacrylamide gel. We are then able to quantitate the radioactivity of appropriate bands utlizing a phosphorimager. RNA from positive and negative control cell lines are used to optimally adjust the PCR
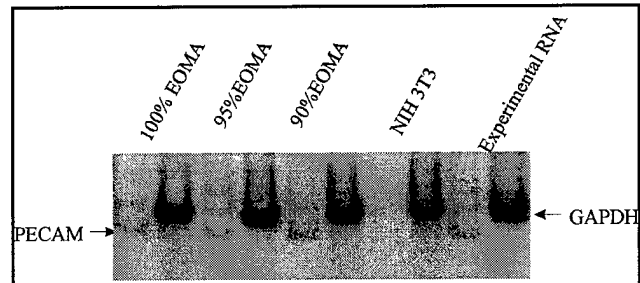

Figure 4. RNA purified from isolated endothelial cells contains transcripts for murine PECAM-1. RT reactions were performed on each RNA utilizing random hexamers as primers. The resulting cDNA was split equally between two PCR reactions. The first PCR reaction contained primers for murine PECAM and the second PCR reaction contained primers for murine GAPDH. The reactions from the same RNA are run in adjacent lanes as follows: Lanes 1 & 2 - 100% EOMA murine hemangioma cells; Lanes 3 & 4 - 95% EOMA cells, 5% NIH 3T3 murine fibroblasts; Lanes 5 & 6 - 90% EOMA cells, 10% NIH 3T3 cells; Lanes 7 & 8 - 100% NIH 3T3 cells; Lanes 9 & 10 - Experimental RNA from the isolated cells. Ratios of the radioactive bands for PECAM and GAPDH were calculated in each instance. The experimental RNA had a higher PECAM/GAPDH ratio than 100% EOMA RNA.
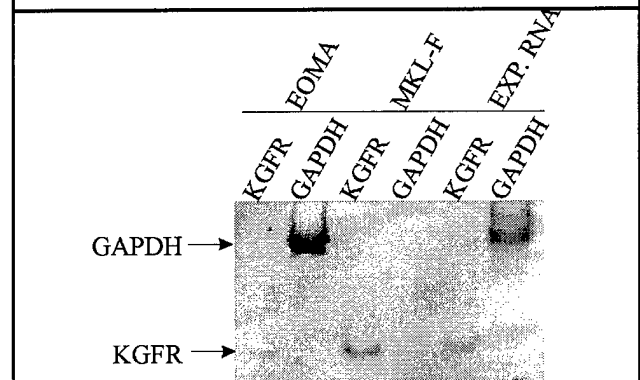

Figure 5. RT-PCR analysis of RNA from tumor-derived endothelial cells reveals small amounts of contaminating transcripts from human tumor cells. RT reactions were run on each RNA and the resultant cDNA split between two tubes as above. One of the tubes was used in a PCR reaction for human KGF receptor and the second for murine GAPDH. Lanes 1 & 2 contain PCR products from EOMA murine hemangioma cells, lanes 3 and 4 contain PCR products from MKL-F human breast carcinoma cells which were used to produce the tumor from which the endothelial cells were isolated, and lanes 5 & 6 contain PCR products from RNA of endothelial cells isolated from the tumor. It can be seen that this experimental RNA contains a few transcripts of human KGF receptor, since a light band is present in lane 5.

10

conditions so that we obtain robust, logarithmic PCR amplification for each set of primers. We have also developed mouse-specific primers for glyceraldehyde 3-phosphate dehydrogenase (GAPDH), a "housekeeping" gene which is constitutively expressed in all mouse cells. Using these techniques, we were able to demonstrate that RNA isolated from the sorted cells contained transcripts for PECAM-1 in a greater abundance than that of a cultured murine hemangioma cell line, EOMA, indicating that the majority of the cells which contributed to the RNA expressed this transcript at high levels (Figure 4). (Note that the PECAM primers do not produce a band when RNA from NIH 3T3 cells, a murine fibroblast line which does not express this mRNA, is used.) In order to detect RNA derived from contaminating human tumor cells, we have utilized primers for the human keratinocyte growth factor receptor (KGFR), which is present in the human tumor cells but should not be present on mouse endothelial cells. This transcript was detected in RNA from the previously isolated endothelial cells (Figure 5), indicating that some tumor cells contaminated the sorted cells. For that reason, we wished to enhance our FACS procedure to more selectively sort out endothelial cells only. In the interval, a third monoclonal antibody for murine PECAM-1 has become available. This antibody was produced in hamster and is not directly coupled to a fluorochrome, necessitating the use of a fluorescently-tagged anti-hamster secondary antibody in the FACS procedure. We have tried out this procedure using a mixture of cultured EOMA cells and the MKL-F tumor cells and find that we do get an enhancement of fluorescence with addition of the third anti-PECAM-1 antibody. Since the new procedure does given enhanced fluorescence, it theoretically will enhance our ability to purify endothelial cells, so we will retain it in the protocol.

We have spent much of the past year searching for a gene which is expressed in mouse fibroblasts but not in endothelial cells. Possibly because of the common embryonic origin of fibroblasts and endothelial cells, many genes seem to be expressed in both cell types. We have identified a gene, keratinocyte growth factor (KGF) which is expressed in NIH 3T3 mouse fibroblasts but not in endothelial-like mouse EOMA cells. We are currently in the process of perfecting a RT-PCR assay for this transcript so we can estimate the degree of fibroblast contamination in our sorted cells.

We are currently producing tumors in nude mice utilizing the parental MCF-7 cells and the transfected cell lines. We have begun isolating mammary fat pad endothelial cells from mice. Therefore, we expect to have RNA in hand by the end of the summer to begin our differential cloning efforts which will identify genes selectively expressed by endothelial cells participating in tumor-induced angiogenesis. RNA obtained from these efforts will also be utilized to identify particular FGF or VEGF receptors important in tumor-induced angiogenesis, below.

As part of Aim 3, FGF receptor gene expression was to be analyzed in endothelial cells by *in situ* hybridization. It would be expected that receptors activated by the particular ligands expressed by the tumor cells (FGF-1 or FGF-4) would be up or downregulated in response to the chronic state of activation and that the particular FGF receptors important in stimulating

angiogenesis could be identified on that basis. FGF receptors are encoded by four genes and have a common structure, with two or three extracellular immunoglobulin loops, a transmembrane domain and a cytoplasmic split kinase domain (2). A complicating feature of FGF receptors is the presence of multiple isoforms of each of the proteins encoded by three of the four FGF receptor genes. The isoforms are produced by mRNA splice variants and may have differing affinities for different ligands or even be kinase defective and/or secreted (2-5). Since the proposal was written, knowledge has been gained about ligand specificity of the various FGF receptors and isoforms, about homo- and heterodimerization between them, and about downstream signal transduction pathways (2,6), underscoring the importance of the data we propose to obtain. Moreover, since we are expanding our studies to include VEGF, it would be appropriate to also include the VEGF receptors, FLT-1 and KDR (7), in the analysis. Therefore, since the original proposal was written almost four years ago, we feel it is appropriate to revisit the planning of this part of the project in order to gain the most appropriate data in light of updated knowledge.

Because *in situ* hybridization is a labor-intensive activity which also consumes expensive laboratory supplies and needs considerable optomization for each probe utilized, this technique may not be appropriate for the large number of isoforms/receptors we wish to analyze. Moreover, there is a controversy in the literature concerning FGF receptors on vascular endothelium because in spite of a vigorous *in vivo* angiogenic response to FGFs in many model systems, several studies have been unable to demonstrate FGF receptor mRNA on microvessels by *in situ* hybridization techniques (8-11). However, a few investigators have shown FGF receptors to be present on tumor endothelium either by *in situ* hybridization (12), or immunohistochemistry (13). Binding studies have shown FGF receptors on quiescent cultured endothelial cells to be present in low copy number (14,15), implying that *in situ* hybridization techniques, at least as some have used them, may not be sensitive enough to detect the mRNA in vascular endothelium *in vivo*. In addition, FGF receptors are rather ubiquitously expressed by many cell types, so that if a signal were obtained from the small number receptors present on endothelial cells in a tissue section by *in situ* hybridization, it might be overwhelmed by signal from neighboring cells. Therefore, we propose to first analyze FGF receptor and VEGF receptor expression by RT-PCR in endothelial cell RNA obtained from the FACS-sorted cells, above. FGF receptor isoforms analyzed will be those which have differing ligand binding properties. These include splice variants of FGF receptors 1-3, all of which have alternative exons (IIIb or IIIc) in the third immunoglobulin loop. In addition, for FGF receptors 1-3, we will analyze for the presence or absence of immunoglobulin loop I, which is also important in determining ligand specificity of the isoform. Isoforms of FGF receptor 4 have not been characterized (2), so only the full-length receptor will be analyzed. RT-PCR conditions will be optomized, as they were for murine PECAM-1, human KGFR, and murine KGF, above, so that robust, logarithmic amplification is obtained. Control cell lines which express the various receptors will be used to standardize PCR conditions. We will then have semiquantitative RT-PCR assays, which can be used to compare levels of expression among the various endothelial cell populations isolated from the tumors or mammary fat pads. The same procedures will be used to assay for VEGF receptors. If particular

FGF receptor isoforms or VEGF receptors are found to be up or down regulated in endothelial cells isolated from tumors produced by control ML-20 cells or the FGF-1, FGF-4, or VEGF transfected cells when compared with mammary fat pad endothelial cells, we will attempt to confirm these results by *in situ* hybridization of the pertinent tumors or mammary fat pad. Probes for the specific isoform/receptor can easily be contructed with the PCR product used for RT-PCR analysis.

We have begun developing RT-PCR assays for some of the FGF receptors/isoforms. So far, we have primers which will identify isoforms of murine FGF receptor 1 which lacks or has immunoglobulin loop I, or has one of the two alternative exons, IIIb or IIIc. In addition, we have primers for the same isoforms of murine FGF receptor 2 and 3. These assays are now being optomized using murine cell lines which are known to express the receptors in question. Figure 6 shows an RT-PCR assay we have adapted from the literature (16) for FGF receptor 1 which compares the
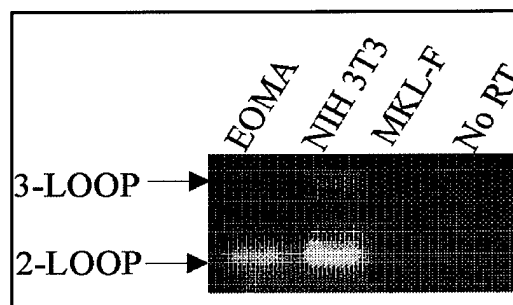

Figure 6. PCR primers which identify murine 3-loop or 2-loop FGF receptor 1 are mouse-specific and do not identify human FGF receptor 1. Lanes 1 & 2 contain RT-PCR reactions using RNA from murine EOMA and murine NIH 3T3 cells. Bands representing both the 3-loop and the 2-loop FGF receptor 1are present, although the 2-loop isoform is more abundant. The 3-loop isoform is more abundant in the NIH 3T3 cells than the EOMA. The primers utilized do not reveal a band in the lane using RNA from human MKL-F cells even though these cells express the human form of this receptor. No RT = no reverse transcriptase, a control for genomic DNA (RNA from the EOMA cell line was used).

mRNAs present for the 3-loop receptor vs the 2-loop receptor which lacks immunoglobulin loop I. It can be seen that both mouse cell lines, EOMA hemangioma cells and NIH 3T3 fibroblasts, express both isoforms, but the 2-loop variety is much more abundant. NIH 3T3 cells express more of the 3-loop isoform than do EOMA cells. The primers do not identify FGF receptor 1 in human MKL-F cells, in spite of the fact that it is expressed by these cells (unpublished), since they are mouse-specific. We have primers which identify other isoforms of FGF receptor 1 as well as primers which identify various isoforms of FGF receptors 2-4, and are in the process of optomizing PCR conditions for these assays. Thus, when we obtain the RNA from tumor-induced endothelial cells or mammary fat pad endothelial cells as described above, we will be in a position to compare the different FGF receptors/isoforms among them. Similar assays will be developed for VEGF receptors. We hope to submit a manuscript with the method of endothelial cell isolation and the FGF and VEGF receptor data by the end of the year.

13

CONCLUSION

Scientific conclusions to date:

1. BrdU incorporation may be a good *in situ* indicator of tumor growth in this model system, which will enable us to identify rapidly or slugglishly growing tumors at time points before differences in tumor size are apparent.

2. Apoptosis as revealed by TUNEL staining is not indicative of tumor growth in this model, perhaps because there is a very low rate of apoptosis, making observations subject to high variability.

3. Edge-associated microvessel density and intratumoral vessel density seem to be postively associated with BrdU incorporation and normal stromal vessel density seems to be negatively associated. Further work is needed to clarify these relationships with respect to statistical significance.

4. RNA purified from endothelial cells isolated from growing tumors is greatly enriched for endothelial cells but still contains tumor cell contamination. New techniques may result in greater purity of isolated endothelial cells.

Discussion:

Microvessel density has been shown in many studies to be a powerful predictor of prognosis in node-negative breast cancer (reviewed in (17)). However, other studies utilizing the same or similar techniques have failed to find a correlation (18-21). It is certainly not at all clear that all breast tumors have similar mechanisms for stimulation of neoangiogenesis. Moreover, identification of tumor-associated microvessels requires a subjective judgement which may exclude informative characteristics because of observer bias. By performing our analyses utilizing tumors produced by cells which have the same genetic background but which express different angiogenic factors, we are in a position to evaluate various angiogenic mechanisms in breast tumors. Such analysis can also include investigation into the influence of estrogen and tamoxifen. Moreover, since the growth characteristics of the tumor cell lines are known, by using an analysis which evaluates microvessels of differing locations or morphologies, we can discern correlations between these microvessels and tumor growth. Thus, it is possible that our research into topography and morphology of tumor-induced blood vessels may shed light on difficulties experienced by others in evaluating micro-vessels in human breast cancer specimens. Our analysis of FGF or VEGF receptor expression may also clarify the situation regarding expression of these receptors in microvessels in human tumors. Our efforts to identify uniquely expressed genes in microvessels associated with productive growth in tumors may also turn out to be useful if such gene expression can be used as a predictor of prognosis. This development would probably precede any use of such uniquely expressed genes as targets for cancer therapy.

Thus, although this project has been subject to a long start-up time which allowed for the development of new techniques, it would seem that we are now on the threshold of our most productive period, in which new data can be generated which will be highly significant for the field of tumor-induced angiogenesis. It can be hoped that others will apply the techniques we have developed in ways which will further the state of scientific knowledge and result in new approaches to breast cancer.

REFERENCES

1. Gavrieli, Y., Sherman, Y., and Ben-Sasson, S.A. Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. J. Cell Biol. *119*: 493-501, 1992.

2. Green, P.J., Walsh, F.S., and Doherty, P. Promiscuity of fibroblast growth factor receptors. Bioessays, *18*: 639-646, 1996.

3. Basilico, C. and Moscatelli, D. The FGF family of growth factors and oncogenes. Adv. Cancer Res. *59*: 115-165, 1992.

4. Givol, D. and Yayon, A. Complexity of FGF receptors: genetic basis for structural diversity and functional specificity. FASEB J. *6*: 3362-3369, 1992.

5. Johnson, D.E. and Williams, L.T. Structural and functional diversity in the FGF receptor multigene family. Adv. Cancer Res. *60*: 1-41, 1993.

6. Zimmer, Y., Givol, D., and Yayon, A. Multiple structural elements determine ligand binding of fibroblast growth factor receptors - Evidence that both IG domain-2 and domain-3 define receptor specificity. J. Biol. Chem. *268*: 7899-7903, 1993.

7. Dvorak, H.F., Brown, L.F., Detmar, M., and Dvorak, A.M. Vascular permeability factor/vascular endothelial growth factor, microvascular hyperpermeabiliity, and angiogenesis. Am. J. Pathol. *146*: 1029-1039, 1995.

8. Millauer, B., Wizigmann-Voos, S., Schnürch, H., Martinez, R., Moller, N.P.H., Risau, W., and Ullrich, A. High affinity VEGF binding and developmental expression suggest Flk-1 as a major regulator of vasculogenesis and angiogenesis. Cell, *72*: 835-846, 1993.

9. Peters, K.G., Werner, S., Chen, G., and Williams, L.T. Two FGF receptor genes are differentially expressed in epithelial and mesenchymal tissues during limb formation and organogenesis in the mouse. Development, *114*: 233-243, 1992.

10. Wanaka, A., Milbrandt, J., and Johnson, E.M., Jr. Expression of FGF receptor gene in rat development. Development, *111*: 455-468, 1991.

11. Heuer, J.G., von Bartheld, C.S., Kinoshita, Y., Evers, P.C., and Bothwell, M. Alternating phases of FGF receptor and NGF receptor expression in the developing chicken nervous system. Neuron, *5*: 283-296, 1990.

12. Leung, H.Y., Hughes, C.M., Klöppel, G., Williamson, R.C.N., and Lemoine, N.R. Localisation of expression of fibroblast growth factors and their receptors in pancreatic adenocarcinoma by *in situ* hybridisation. Int. J. Oncol. *4*: 1219-1223, 1994.

13. Morikawa, Y., Ishihara, Y., Tohya, K., Kakudo, K., Seo, M.K., and Matsuura, N. Expression of the fibroblast growth factor receptor-1 in human normal tissues and tumors determined by a new monoclonal antibody. Arch. Pathol. Lab. Med. *120*: 490-496, 1996.

14. Moscatelli, D. Metabolism of receptor-bound and matrix-bound basic fibroblast growth factor by bovine capillary endothelial cells. J Cell Biol, *107*: 753-759, 1988.

15. Moscatelli, D. High and low affinity binding sites for basic fibroblast growth factor on cultured cells: Absence of a role for low affinity binding in the stimulation of plasminogen activator production by bovine capillary endothelial cells. J Cell Physiol, *131*: 123-130,

1987.

16. Jin, Y., Pasumarthi, K.B.S., Bock, M.E., Lytras, A., Kardami, E., and Cattini, P.A. Cloning and expression of fibroblast growth factor receptor-1 isoforms in the mouse heart; evidence for isoform switching during heart development. J. Mol. Cell Cardiol. *26*: 1449-1459, 1994.

17. Weidner, N., Semple, J.P., Welch, W.R., and Folkman, J. Tumor angiogenesis and metastasis - correlation in invasive breast carcinoma. N. Engl. J. Med. *324*: 1-8, 1991.

18. Van Hoef, M.E.H.M., Knox, W.F., Dhesi, S.S., Howell, A., and Schor, A.M. Assessment of tumour vascularity as a prognostic factor in lymph node negative invasive breast cancer. Eur. J. Cancer, *29A*: 1141-1145, 1993.

19. Page, D.L. and Jensen, R.A. Angiogenesis in human breast carcinoma: What is the question? Hum. Pathol. *26*: 1173-1174, 1995.

20. Costello, P., McCann, A., Carney, D.N., and Dervan, P.A. Prognostic significance of microvessel density in lymph node negative breast carcinoma. Hum. Pathol. *26*: 1181-1184, 1995.

21. Goulding, H., Nik Abdul Rashid, N.F., Robertson, J.F., Bell, J.A., Elston, C.W., Blamey, R.W., and Ellis, I.O. Assessment of angiogenesis in breast carcinoma: An important factor in prognosis? Hum. Pathol. *26*: 1196-1200, 1995.

McLeskey, Sandra W.
DAMD17-94-J4173

APPENDIX

LIST OF ABBREVIATIONS

a.k.a.   Also known as
BrdU   Bromodeoxyuridine, a thymidine analog
EOMA   A mouse hemangioma cell line
FACS   Fluorescence activated cell sorting
FGF   Fibroblast growth factor
FGF-1   Fibroblast growth factor 1, a.k.a. acidic FGF
FGF-1, clone 18 A clonal cell line of ML-20 cells (below) transfected with FGF-1
FGF-4   Fibroblast growth factor 4, a.k.a. Kaposi FGF, *hst-1*
FGFR   FGF receptor
KGFR   Keratinocyte growth factor receptor
lacZ   A bacterial gene encoding β-galactosidase
MCF-7   An estrogen receptor positive breast carcinoma cell line
MKL-F   A clonal cell line of ML-20 cells cotransfected with FGF-4 and *lacZ*
MKL-4   A clonal cell line of MCF-7 cells cotransfected with FGF-4 and *lacZ*
ML-20   A clonal cell line of MCF-7 cells transfected with *lacZ*
NIH 3T3   A cell line of immortalized mouse fibroblasts
PECAM   Platelet-endothelial cell adhesion molecule
RT-PCR   Reverse transcription followed by the polymerase chain reaction
TUNEL   Terminal deoxynucleotidyl transferase - mediated dUTP-biotin nick end labeling
VEGF   Vascular endothelial cell growth factor

Meeting abstracts since the last report:

El-Ashry, D., Tobias, C.A., and **McLeskey, S.W.**, Two clonal FGF-4 transfected MCF-7 cell lines with different hormonal growth responses *in vivo* have differing responses to hormonal manipulation. 88th Annual Meeting of the American Association for Cancer Research, San Diego, CA., April 12-16, 1997.

Bullocks, J., Zhang, L., Ding, I.Y.F., **McLeskey, S.W.**, Tobias, C.A., Miller, D.L., and Kern, F.G. Overexpression of vascular endothelial growth factor (VEGF) in MCF-7 breast carcinoma cells facilitates growth in tamoxifen-treated nude mice and tumor cell dissemination. 88th Annual Meeting of the American Association for Cancer Research, San Diego, CA, April 12-16, 1997.

Publications since the last report:

Zhang, L., Kharbanda, S., Chen, D., Bullocks, J., Millder, D.L., Ding, I.Y.F., Hanfelt, J., **McLeskey, S.W.**, and Kern, F.G. MCF-7 breast carcinoma cells overexpressing FGF-1 form vascularized, metastatic tumors in ovariectomized or tamoxifen-treated nude mice. 1997, Oncogene, in press.

Manuscripts in preparation:

Low, J.A., Holst-Hansen, C., Brünner, N., Tobias, C., **McLeskey, S.W.**, Bone, E.A., Johnson, M.D., and Dickson, R.B. Increased stromal expression of urokinase plasminogen activator in a human breast tumor xenograft model following anti-MMP therapy. 1997, *Clin. Exp. Metastasis*, submitted.

McLeskey, S.W., Zhang, L., Trock, B. J., Lopez, C.A., Kharbanda, S., Tobias, C. A., El-Ashry, D., Dickson, R. B. and Kern, F.G. Tamoxifen-resistant fibroblast growth factor transfected MCF-7 cells are cross-resistant *in vivo* to the antiestrogen, ICI 182,780, and two aromatase inhibitors. *Clin. Cancer Res.,* submitted.

Shiao,R.-T., Tao,X.-C., Khera,S.Y., **McLeskey, S.W.**, Zwiebel, J.A., Kern, F.G., and Freter, C.E. Overexpression of interleukin-6 in MCF-7 breast cancer cells increases tumorigenicity in nude mice. Manuscript in revison.

Tobias, C. A., Bryan, J. A., Vezza, P. R., Filie, A. F., and **McLeskey, S. W.** A model of tumor-induced angiogenesis. Manuscript in preparation.

Personnel receiving pay from this negogiated effort:

Sandra W. McLeskey, PhD